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Correlation of Neo-Sensitabs Tablet Diffusion Assay Results on Three Different Agar Media with CLSI Broth Microdilution M27-A2 and Disk Diffusion M44-A Results for Testing Susceptibilities of *Candida* spp. and *Cryptococcus neoformans* to Amphotericin B, Caspofungin, Fluconazole, Itraconazole, and Voriconazole[▽]

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We compared the Neo-Sensitabs tablet assay to both reference M27-A2 broth microdilution and M44-A disk diffusion methods for testing susceptibilities of 110 isolates of *Candida* spp. and *Cryptococcus neoformans* to amphotericin B, caspofungin, fluconazole, itraconazole, and voriconazole. Neo-Sensitabs assay inhibition zone diameters in millimeters on three agars (Mueller-Hinton agar supplemented with 2% dextrose and 0.5 μg/ml methylene blue [MGM], Shadomy [SHA], and RPMI 1640 [RPMI, 2% dextrose]) were obtained at 24 to 72 h. The correlation coefficient of Neo-Sensitabs results with MICs was similar to that of the disk method for most of the five agents on MGM (*R*, 0.80 to 0.89 versus 0.76 to 0.89, respectively). Overall, superior correlation was observed at 24 h for most agents. The exception was amphotericin B (*R* values of 0.68 and 0.5 for disk and tablet, respectively, at 48 h versus 0.68 and 0.48, respectively, at 24 h). In general, Neo-Sensitabs results were less consistent on SHA and RPMI agars. Although agreement by breakpoint category of Neo-Sensitabs and disk results with CLSI method M27-A2 was also similar on MGM (92.7 to 98.2% versus 95.5 to 100%, respectively), the Neo-Sensitabs method failed to identify two of the six isolates with high amphotericin B MICs. These data suggest the potential value of the Neo-Sensitabs assay for testing at least four of the five agents against yeasts evaluated in the clinical laboratory.

The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed reproducible procedures for antifungal susceptibility testing of yeasts by broth microdilution (document M27-A2) and disk diffusion (document M44-A for fluconazole and voriconazole) methods (4–7). The Neo-Sensitabs assay (A/S Rosco Diagnostica, Taastrup, Denmark) utilizes a 9-mm-diameter (1-mm-thickness) tablet for antimicrobial susceptibility testing including several antifungal agents (3). This assay has been favorably investigated for testing yeasts with 15-μg fluconazole tablets on either Shadomy (SHA) or RPMI 1640 agar (2, 21, 22) and more recently with posaconazole (9). Amphotericin B, fluconazole, itraconazole, and voriconazole tablets (A/S Rosco Diagnostica) and 6-mm disks (Abtek Biologicals Ltd., Liverpool, United Kingdom) are available in Europe but are not yet available in the United States.

The purpose of this study was to compare the Neo-Sensitabs tablet assay to both CLSI reference broth microdilution (document M27-A2) and disk diffusion (document M44-A) methods for testing susceptibilities of 10 to 20 isolates each of *Candida* (eight species) and *Cryptococcus neoformans* to five antifungal agents (amphotericin B, caspofungin, fluconazole, itraconazole, and voriconazole). The evaluation included the following determinations: (i) determination of reference MICs of the five agents by CLSI broth microdilution method M27-A2, (ii) determination of inhibition zone diameters (in millimeters) by the reference M44-A disk diffusion method and by commercial Neo-Sensitabs tablet diffusion methods, (iii) determination of the correlation coefficient between inhibition zone diameters (in millimeters) and reference MICs of the five antifungal agents, and (iv) determination of the agreement by breakpoint category of disk and tablet inhibition zone diameters (in millimeters) with reference MICs of the five agents.

MATERIALS AND METHODS

Isolates. A total of 110 isolates of *Candida* spp. and *Cryptococcus neoformans* were evaluated by each method. The set of isolates included strains with different patterns of susceptibility to caspofungin and amphotericin B as well as fluconazole-, voriconazole-, and itraconazole-resistant, -susceptible-dose-dependent (S-DD), and -susceptible isolates (Table 1). The set of isolates also included five reference isolates recommended for amphotericin B testing (*Candida albicans* ATCC 200955 [resistant], *Candida lusitaniae* ATCC 200950 and ATCC 200951 [resistant], *Candida parapsilosis* ATCC 200954 [susceptible], and *Candida tropicalis* ATCC 200956 [resistant]), three caspofungin-resistant isolates (two laboratory mutants and another provided by M. Pfaller), and the susceptible wild-type *C. albicans* isolate (10, 13). The CLSI quality control (QC) isolates *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were tested each time a set of isolates was evaluated by the three procedures; MICs of each antifungal agent for both QC isolates were within the established MIC limits (6).

CLSI broth microdilution procedure (document M27-A2). MICs of fluconazole (Pfizer Central Research, New York, NY), itraconazole (Janssen, Beerse, Belgium), voriconazole (Pfizer Central Research), amphotericin B (Bristol-Myers Squibb Pharmaceuticals Research Institute, Wallingford, CT), and caspofungin (Merck Research Laboratories, Rahway, NJ) were determined by the CLSI M27-A2 broth microdilution method (4). Final drug concentrations ranged

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TABLE 1. Set of isolates evaluated and their available well-documented in vitro data in this and other studies[a]

Species (total no. of isolates)	Agent	No. of isolates categorized as:		
		S	S-DD	R
C. albicans (20)	Fluconazole	5	4	11
	Voriconazole	19	1	0
	Itraconazole	11	6	3
	Amphotericin B	18	NA	2[b]
	Caspofungin	17[c]	NA	3[d]
C. dubliniensis (10)	Fluconazole	10	0	0
	Voriconazole	10	0	0
	Itraconazole	10	0	0
	Amphotericin B	10	NA	0
	Caspofungin	10	NA	0
C. glabrata (10)	Fluconazole	6	0	4
	Voriconazole	7	0	3
	Itraconazole	4	2	4
	Amphotericin B	10	NA	0
	Caspofungin	10	NA	0
C. guilliermondii (10)	Fluconazole	8	0	2
	Voriconazole	8	2	0
	Itraconazole	5	3	2
	Amphotericin B	10	NA	0
	Caspofungin	10	NA	0
C. krusei (10)	Fluconazole	0	8	2
	Voriconazole	10	0	0
	Itraconazole	0	8	2
	Amphotericin B	9	NA	1
	Caspofungin	10	NA	0
C. lusitaniae (10)	Fluconazole	10	0	0
	Voriconazole	10	0	0
	Itraconazole	8	2	0
	Amphotericin B	8	NA	2[b]
	Caspofungin	10	NA	0
C. parapsilosis (10)	Fluconazole	10	0	0
	Voriconazole	10	0	0
	Itraconazole	8	2	0
	Amphotericin B	10	NA	0
	Caspofungin	10	NA	0
C. tropicalis (10)	Fluconazole	9	0	1
	Voriconazole	10	0	0
	Itraconazole	8	0	2
	Amphotericin B	9	NA	1[b]
	Caspofungin	10	NA	0
Cryptococcus neoformans (20)	Fluconazole	19	1	0
	Voriconazole	20	0	0
	Itraconazole	20	0	0
	Amphotericin B	20	NA	0
	Caspofungin	0	NA	20
Total (110)	Fluconazole	77	13	20
	Voriconazole	104	3	3
	Itraconazole	74	23	13
	Amphotericin B	104	NA	6
	Caspofungin	87	NA	23

[a] Isolates were grouped according to CLSI breakpoints for fluconazole, voriconazole, and itraconazole as being susceptible (S), susceptible-dose dependent (S-DD), and resistant (R). Amphotericin B and caspofungin MICs of ≤1 μg/ml were categorized as being susceptible, and those of ≥2 μg/ml were categorized as being resistant or with reduced susceptibility; most other isolates were evaluated in collaborative studies. NA, not applicable.

[b] Includes proven amphotericin B-resistant (animal model) isolates (10).

[c] Includes the wild-type caspofungin-susceptible isolate (13).

[d] Includes two caspofungin-resistant laboratory mutants and one isolate from M. Pfaller (13).

from 128 to 0.25 μg/ml (fluconazole) and 16 to 0.03 μg/ml (other agents). Microdilution trays containing 100 μl of twofold serial dilutions of the antifungal drugs in standard RPMI 1640 broth were inoculated with 100 μl of the diluted inoculum containing between 1.0×10^3 and 5×10^3 CFU/ml; microdilution trays were incubated in ambient air at 35°C for 24 to 48 h (*Candida* spp.) and 72 h (*C. neoformans*), and caspofungin MICs were determined at 24 h (*Candida* spp.) and 72 h (*C. neoformans*) (4, 13). Reference MICs were defined as the lowest drug concentrations that showed either ≥50% (triazoles and caspofungin) or 100% (amphotericin B) growth inhibition compared with the growth control (4). QC isolates were tested in the same manner.

In addition, isolates for which amphotericin B MICs were >1 (including the amphotericin B-resistant reference isolates listed above) and five isolates of each species for which MICs were ≤1, including the susceptible reference *C. parapsilosis* ATCC 200954 isolate, were tested by Etest (10).

CLSI disk diffusion procedure (document M44-A). Although the CLSI M44-A document describes guidelines for fluconazole and voriconazole only (5), we followed these guidelines for disk testing of these two agents and with certain modifications for the other agents. Mueller-Hinton agar supplemented with 2% dextrose and 0.5 μg/ml methylene blue (MGM; Hardy Diagnostics, Santa Maria, CA) (150-mm plates) was inoculated with the undiluted inoculum (0.5 McFarland standard). Ten-microgram amphotericin B and itraconazole (Abtek Biologicals Ltd.), 25-μg fluconazole, 1-μg voriconazole (Becton Dickinson and Company, Sparks, MD), and 5-μg caspofungin disks were applied to the inoculated agar; two types of caspofungin disks were evaluated (COD [Becton Dickinson] and CBD [Oxoid Limited, Basingstoke, Hampshire, England]). The plates were incubated in ambient air at 35°C. QC isolates were tested in the same manner.

Neo-Sensitabs tablet assay. The Neo-Sensitabs tablet assay was performed according to the manufacturer's instructions (Neo-Sensitabs user's guide; A/S Rosco Diagnostica, Taastrup, Denmark) and M44-A guidelines (5). Briefly, MGM (Hardy Diagnostics) agar plates (150 mm) were inoculated as described above, and 9-mm tablets (amphotericin B [10 μg], caspofungin [5 μg], fluconazole [25 μg], itraconazole [8 μg], and voriconazole [1 μg]) provided by Rosco Laboratory (A/S Rosco Diagnostica) were evaluated. The plates were incubated in ambient air at 35°C. QC isolates were tested in the same manner. In addition to MGM agar, each isolate was inoculated onto Shadomy (A/S Rosco Diagnostica) (3) and RPMI 1640 (RPMI plus 2% dextrose; Remel, Lenexa, KS) agar plates.

Inhibition zone diameter determination. Zone diameters by both disk and tablet diffusion assays were measured to the nearest whole millimeter at a point in which there was either a prominent reduction of growth (80% for triazoles and caspofungin) or no visible growth (clear zones for amphotericin B) after 24 and 48 h (*Candida* spp.) and 48 to 72 h (*C. neoformans*); zone diameter results with fluconazole and voriconazole by the disk methodology were read only at 24 h as described by CLSI document M44-A (5).

Reproducibility methodology. Inhibition zone diameters were obtained on three different days for selected isolates (low and high reference MICs) for 32 of the 110 yeasts evaluated (9).

Data analysis. For the correlation between reference MICs and inhibition zone diameters (in millimeters) around the disks and tablets, a linear regression analysis using the least-squares method (Pearson's correlation coefficient; MS Excel software) was performed by plotting zone diameters against their respective MIC endpoints (9). The reproducibility of zone diameters obtained on different days with selected study isolates was evaluated by calculating the percentage of replicate zone diameters that were within 2 standard deviations from the mean (9).

Available interpretive CLSI MIC breakpoints (fluconazole, voriconazole, and itraconazole, defined as susceptible [≤8, ≤1, and ≤ 0.12 μg/ml, respectively], S-DD [16 to 32, 2, and 0.25 to 0.5 μg/ml, respectively], and resistant [≥64, ≥4, and ≥1 μg/ml, respectively]) were used to analyze the agreement by breakpoint category (6, 7) as follows: (i) agreement by breakpoint category between inhibition zone diameters (disk and tablet on MGM agar) and 24-h (caspofungin) and 48-h (other agents) reference MICs and (ii) tablet zone diameters on SHA and RPMI agars and reference MICs (with the same incubation times described above). Very major errors were identified when the reference MIC was resistant and the result was susceptible by either tablet or disk. Major errors were identified when the isolate was classified as being resistant by either tablet or disk and as being susceptible by the reference MIC; minor errors corresponded to shifts between susceptible and S-DD or S-DD and resistant. Interpretive criteria are not available for either amphotericin B or caspofungin. Caspofungin MIC profiles indicate that ≥99% of *Candida* species isolates are inhibited by ≤1 μg/ml and MICs of >8 μg/ml are obtained for *C. neoformans* (a species that is recognized as being resistant to caspofungin) (8, 13, 14, 16). Traditionally, isolates with

TABLE 2. Comparison of QC strain zone diameters by CLSI M44-A disk method versus Neo-Sensitabs assay on three different media[a]

QC isolate	Agent	Zone diam limit (mm)		Tablet zone diam range (mm) on:		
		Reference	This study (on MGM agar)	MGM	RPMI	SHA
C. parapsilosis ATCC 22019	Fluconazole	22–33[b]	22–30	25–29	35–39	17–18
	Voriconazole	28–37[b]	29–36	28–33	39–40	28–30
	Itraconazole	28–35[c]	19–25	24–25	29–30	25–26
	Amphotericin B	20–26[c]	21–24	20–26	20–21	18–20
	Caspofungin	13–25[d]	15–25	11–18	11–15	12–18
C. krusei ATCC 6258	Fluconazole	NA	12–14	12–15	18–20	11–15
	Voriconazole	16–25[b]	20–22	24–25	33–34	23–25
	Itraconazole	16–22[c]	16–22	21–22	25–27	19–22
	Amphotericin B	15–21[c]	15–21	19–21	18–21	16–21
	Caspofungin	13–25[d]	16–22	17–19	12–15	16–18

[a] Tests were performed more than three times with MGM, RPMI, and SHA agars. NA, not applicable.
[b] CLSI fluconazole and voriconazole zone diameter limits (5). Disk and tablet doses were as follows: 25 μg (fluconazole); 1 μg (voriconazole); 10 and 8 μg, respectively (itraconazole); 10 μg (amphotericin B); and 5 μg (caspofungin).
[c] Rosco zone diameter limits on SHA agar.
[d] Combined zone diameter limits obtained with the two disks (Oxoid and Becton Dickson) in a previous collaborative CLSI study.

amphotericin B MICs that are >1 μg/ml are considered to be resistant, especially when these results are obtained by the Etest. Based on those susceptibility patterns, we tentatively grouped the isolates with MICs of ≤1 μg/ml as being susceptible and those with MICs of ≥2 μg/ml as being resistant to analyze the agreement by breakpoint category between inhibition zone diameters and MICs for these two antifungal agents.

RESULTS AND DISCUSSION

We have evaluated for the first time the suitability of the Neo-Sensitabs tablet assay for testing fluconazole, voriconazole, itraconazole, caspofungin, and amphotericin B against *Candida* spp. and *C. neoformans*. Also, for the first time, we have evaluated the suitability of commercial itraconazole and caspofungin disks. Rosco has developed an agar diffusion assay by using Neo-Sensitabs tablets and SHA agar. The Rosco user's guide has provided interpretive guidelines on both SHA and RPMI agars (Neo-Sensitabs user's guide; A/S Rosco Diagnostica, Taastrup, Denmark). On the other hand, the CLSI has recommended MGM agar as the reference medium for yeast disk testing (5). Because of that, we have investigated the suitability of this commercially available assay using SHA, RPMI, and MGM agars. Although the CLSI disk diffusion method (document M44-A) for testing fluconazole and voriconazole (5) has been available for some years, disks are not commercially available for testing clinical isolates in the United States.

The reproducibility of the Neo-Sensitabs assay with most of the five antifungal agents was similar to that of the disk diffusion method (87 to 98% versus 89 to 97%, respectively, within 2 standard deviations). The highest percentages of agreement were at 24 h for *Candida* spp. and at 72 h for *C. neoformans*; slightly lower percentages of reproducibility were obtained with itraconazole. These reproducibility results were similar to those recently reported for testing posaconazole against molds and yeasts on MGM agar (9) and to those reported in previous CLSI studies (4, 5).

Table 2 provides disk and Neo-Sensitabs method results for two CLSI QC isolates with the five antifungal agents. In addition to reference QC zone limits (5), tentative caspofungin, obtained in a recent collaborative study with two disks (COD and CBD) (unpublished data), amphotericin B, and itraconazole (Neo-Sensitabs user's guide; A/S Rosco Diagnostica) QC zone diameter limits are listed in Table 2. Neo-Sensitabs results for fluconazole and voriconazole on MGM agar were within the expected CLSI limits for QC *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (5), while those on RPMI and SHA agar were outside the limits. Most of our itraconazole and amphotericin B Neo-Sensitabs and disk data were within Rosco's proposed zone diameter limits on MGM agar for both QC isolates (Neo-Sensitabs user's guide; A/S Rosco Diagnostica) but more variable on SHA and RPMI agar. Both caspofungin disk and tablet results using MGM and SHA agar were within the tentative range listed for *C. krusei* ATCC 6258 but outside the range for *C. parapsilosis* ATCC 22019. Therefore, MGM agar provided the most consistent Neo-Sensitabs results for both QC isolates. When reference limits are available for amphotericin B, caspofungin, and itraconazole, the suitability of Neo-Sensitabs should be further determined; a study is in progress regarding the establishment of caspofungin reference zone limits.

Table 3 lists the results of the correlation coefficient (linear regression) analysis between disk or tablet zone diameters and corresponding MICs. MICs read at 24 h (caspofungin for *Candida* spp.) (13), 48 h (other agents for *Candida* spp.), and 72 h (all agents for *C. neoformans*) were plotted against their respective zones of inhibition in millimeters read at 24 and 48 h (*Candida* spp.) and 48 and 72 h (*C. neoformans*). The correlation of the Neo-Sensitabs assay for fluconazole on MGM agar was similar or superior to that of the disk in our study (tablet *R*, 0.889; disk *R*, 0.8768) and previous studies (*R*, 0.6 to 0.7) (17, 19). Results were slightly higher when RPMI agar, which has been a medium recommended by Rosco for Neo-Sensitabs (Neo-Sensitabs user's guide; A/S Rosco Diagnostica), was used. Prior Neo-Sensitabs yeast data have been reported only for fluconazole on SHA agar with a 15-μg disk (21, 22); correlation results in one of those studies were similar (22) to those in our study performed with the 25 μg tablet on SHA agar (*R*, 0.873 and 0.8075, respectively). The 25-μg tablet matches the concentration of the fluconazole disk described in

TABLE 3. Correlation between inhibition zone diameters in millimeters (M44-A disk and Neo-Sensitabs tablet methods) and CLSI document M27-A2 MICs for 110 *Candida* species and *C. neoformans* isolates with five antifungal agents[a]

Agent	Method	Incubation time (h)	*R* value for:		
			MGM	RPMI	SHA
Fluconazole	Disk	24	0.8768	ND	ND
		48	NA	ND	ND
	Tablet	24	0.8891	0.9291	0.8075
		48	0.8650	0.9210	0.7847
Voriconazole	Disk	24	0.7595	ND	ND
		48	NA	ND	ND
	Tablet	24	0.7931	0.7573	0.7365
		48	0.7980	0.7851	0.7096
Itraconazole	Disk	24	0.8648	ND	ND
		48	0.8408	ND	ND
	Tablet	24	0.8515	0.8255	0.8138
		48	0.7946	0.7809	0.7168
Caspofungin	Disk	24	0.8945	ND	ND
		48	0.8036	ND	ND
	Tablet	24	0.8257	0.8176	0.6881
		48	0.7707	0.6881	0.8931
Amphotericin B	Disk	24	0.6811	ND	ND
		48	0.6811	ND	ND
	Tablet	24	0.4778	0.5584	0.5245
		48	0.5032	0.5747	0.5591

[a] Yeasts used were *Candida* spp. (24- and 48-h zone readings versus 24-h [caspofungin] and 48-h [other agents] MICs) and *C. neoformans* (48-h and 72-h zones readings versus 72-h MICs [all agents]); MGM, RPMI (RPMI-1640), and SHA were used. NA, not applicable; ND, not determined.

CLSI document M44-A (5). Data regarding the suitability of the 1-μg voriconazole tablet have not been published. Our results were similar to or slightly higher (24 and 48 h) than those observed by the disk in this (Table 3) and other studies (*R*, 0.7 to 0.96) (17, 18). In general, similar *R* values were obtained for the correlation of Neo-Sensitabs or disk results with amphotericin B, itraconazole, and caspofungin MICs. These results were higher at 24 than at 48 h for itraconazole and caspofungin, as recommended in CLSI document M44-A (5) (Table 3). In contrast, the 48-h incubation improved the correlation for amphotericin B. The latter correlation was also lower than that for the other four agents (tablet and disk). A lack of publications regarding disk or tablet results for itraconazole precluded comparisons, but our *R* values were suitable by both methods. Caspofungin disk and tablet testing provided results (Table 3) that were superior to previously reported results when RPMI agar was used (*R*, 0.59 and 0.53 at 24 and 48 h, respectively). However, that study evaluated noncommercial 2.5-μg caspofungin disks (11). Our correlation results indicated that the three agars performed in a similar manner with most agents; superior results were also obtained when MGM and RPMI were used compared to SHA agar for caspofungin. Therefore, it appears that MGM is a good alternative for Neo-Sensitabs (all agents) and disk (caspofungin, itraconazole, and amphotericin B) testing, which is fortunate, since that is the standard medium for testing of yeasts against fluconazole and voriconazole (5).

The required evaluation of Neo-Sensitabs and disk results regarding breakpoint agreement for the three triazoles is presented in Table 4. CLSI MIC breakpoints and zone interpretive criteria have been established for fluconazole and voriconazole (4, 6, 7). MIC breakpoints have also been established for itraconazole, but guidelines for disk testing are not available. Although triazole MIC breakpoints have been established only for *Candida* spp., breakpoint category analysis has been applied to *C. neoformans* using fluconazole interpretive criteria (19). We have conducted similar analyses for the three triazoles including isolates of this species. Based on itraconazole MIC breakpoints, the following tentative zone category thresholds were assigned to determine agreement (tablet and disk diameters): ≥23 mm (susceptible), 14 to 22 mm (S-DD), and ≤13 mm (resistant). Overall, higher interpretive agreement was observed for the triazoles on MGM (92.7 to 95.5%) than on the other two agars (84.5 to 97.3%). The higher performance of MGM was similar to that reported previously for fluconazole disk testing (20). The interpretive agreement for Neo-Sensitabs was comparable to disk results in this study (95.5 to 96.4%) and previous fluconazole and voriconazole studies (86 to 99.4%) (12, 17–19). Both disk and tablet were able to identify the *C. neoformans* isolate that was S-DD to fluconazole (18-mm zone diameter) as previously reported (19). No very major errors and <10% minor errors were observed only for *Candida* spp. (Table 4). No published data are available for itraconazole. However, both disk and tablet provided suitable results when MGM agar was used, despite the different concentrations of disk and tablet (10 and 8 μg, respectively). The potential for the development of standard guidelines for itraconazole disk testing following the required collaborative studies is indicated. The potential use of the Neo-Sensitabs tablet assay for testing yeasts on the reference MGM agar with the triazoles evaluated is also indicated by these results.

Testing conditions have been identified for caspofungin MIC determinations (13), but neither MIC nor zone diameter breakpoints have been developed for this agent. The availability of resistant *C. albicans* laboratory mutants has made it possible to differentiate isolates with "normal" (or wild type, 99.7% of >8,000 isolates) susceptibility from those with decreased susceptibility (resistant mutants and other isolates) (8, 16). By these testing parameters, our results (Table 5) reflected those previously published findings: most MICs for *Candida* were ≤1 μg/ml, while MICs of ≥2 μg/ml were obtained only for the resistant mutants and isolates of *C. neoformans*. We grouped isolates with MICs of ≤1 μg/ml (corresponding zone diameters of ≥14 mm) as being "susceptible" and MICs of ≥2 μg/ml (corresponding zone diameters of ≤13 mm) as being "resistant" for analyzing disk and tablet data. Of the two 5-μg caspofungin disks, the COD disk (Oxoid) consistently yielded zone diameters of ≤12 mm (including for all *C. neoformans* isolates) for the "resistant" isolates and of 14 to 34 mm for the "susceptible" ones. In contrast, the CBD disk (Becton Dickinson) and Neo-Sensitabs categorized those isolates for which MICs were ≤1 μg/ml as being "resistant" (Table 5). However, the overall level of discrepant values ("major errors") was low (2.7% for CBD disk and 5.5% for tablet on MGM). It is noteworthy that these discrepant results were obtained

TABLE 4. Agreement of reference MICs (CLSI document M27-A2) and zone diameters in millimeters (M44-A disk and Neo-Sensitabs tablet methods) according to CLSI breakpoint categorization for 110 *Candida* species and *C. neoformans* isolates with three antifungal agents

Antifungal agent (no. of isolates tested)	Method-medium[a]	% of MICs by interpretive category[b]			No. of discrepancies (zone diam)	% Error			% Agreement[c]
		S	S-DD	R		Minor	Major	Very major	
Fluconazole (110)	BMD	75.5	11.8	12.7					
	Disk	75.5	8.2	16.3	4	3.6	0	0	96.4
	Tablet-MGM	74.5	9.1	16.4	5	4.5	0	0	95.5
	Tablet-RPMI	76.4	8.2	15.4	4	3.6	0	0	96.4
	Tablet-SHA	68.2	12.7	19.1	12	7.3	3.6	0	89.1
Voriconazole (110)	BMD	94.6	2.7	2.7					
	Disk	93.6	0.9	5.5	5	3.6	0.9	0	95.5
	Tablet-MGM	93.6	0.9	5.5	5	3.6	0.9	0	95.5
	Tablet-RPMI	96.4	0	3.6	3	2.7	0	0	97.3
	Tablet-SHA	94.5	0.9	4.6	5	3.6	0.9	0	95.5
Itraconazole (110)	BMD	68.2	20	11.8					
	Disk	64.5	23.6	11.8	5	4.5	0	0	95.5
	Tablet-MGM	68.2	18.2	13.6	8	7.3	0	0	92.7
	Tablet-RPMI	71.8	18.2	10	14	12.7	0	0	87.3
	Tablet-S HA	70	14.5	15.5	17	15.5	0	0	84.5

[a] BMD, MICs in μg/ml determined at 48 h (*Candida* spp.) and 72 h (*C. neoformans*) by CLSI microdilution method M27-A2; Disk, inhibition zone diameters in mm determined at 24 h (*Candida* spp.) and 72 h (*C. neoformans*) by method M44-A; Tablet-MGM, Tablet-RPMI, and Tablet-SHA, inhibition zone diameters in mm determined at 24 h (*Candida* spp.) and 72 h (*C. neoformans*) by the Neo-Sensitabs tablet method with MGM, RPMI 1640, and Shadomy agars, respectively. Tablets used were 25 μg fluconazole, 1 μg voriconazole, and 8 μg itraconazole; disks used were 25 μg fluconazole, 1 μg voriconazole, and 10 μg itraconazole.

[b] Percentages of MICs and inhibition zone diameters in mm that were within the following CLSI MIC and disk zone interpretive categories: for fluconazole, an MIC of ≤8 μg/ml (≥19 mm) was considered susceptible (S), an MIC of 16 to 32 μg/ml (15 to 18 mm) was considered S-DD, and an MIC of ≥64 μg/ml (≤14 mm) was considered resistant (R); for voriconazole, an MIC of ≤1 μg/ml (≥17 mm) was considered susceptible, an MIC of 2 μg/ml (14 to 16 mm) was considered S-DD, and an MIC of ≥4 μg/ml (<13 mm) was considered resistant. For itraconazole, CLSI MIC breakpoints and study assigned the following zone categories: susceptible, MIC of ≤0.12 μg/ml (≥23 mm); S-DD, MIC of 0.25 to 0.5 μg/ml (14 to 22 mm); R, MIC of ≥1 μg/ml (≤13 mm).

[c] Percentages of inhibition zone diameters in mm compared with the reference MICs that were in agreement regarding the CLSI breakpoint classification.

mostly for *C. parapsilosis* and *C guilliermondii* isolates with MICs of 1 μg/ml. In an animal model of systemic candidiasis, the overall CFU reduction was 100-fold less in mice treated with caspofungin and infected with isolates of these two species than in those infected with *C. albicans* (1). In this study, narrower zone diameters were generally observed for *C. guilliermondii* and especially *C. parapsilosis*; six of the eight isolates with MICs of 1 μg/ml belonged to these two species (data not shown in Table 5). Both caspofungin disk and tablet results were encouraging; "resistant" isolates

TABLE 5. Agreement of reference MICs (method M27-A2) and zone diameters (modified M44-A disk and Neo-Sensitabs tablet methods) according to tentative resistant (MIC ≥2 μg/ml) and susceptible (MIC ≤1 μg/ml) breakpoints for 110 *Candida* species and *C. neoformans* isolates with two antifungal agents

Antifungal agent (no. of isolates tested)	Method-medium[a]	Susceptible isolates[b]			Resistant isolates[b]			% Agreement[c]
		%	Range (mm)	No. of discrepancies	%	Range (mm)	No. of discrepancies	
Caspofungin[d] (110)	BMD	79.1			20.9			
	Disk-COD	79.1	14–34	0	20.9	NZ–12	0	100
	Disk-CBD	76.4	NZ–35	3	23.6	NZ–12	0	97.2
	Tablet-MGM	73.6	NZ–26	6	26.4	NZ–10	0	94.5
	Tablet-RPMI	40.9	NZ–17	42	59.2	NZ	0	60.9
	Tablet-SHA	72.8	11–20	7	27.2	NZ–12	0	93.6
Amphotericin B (110)	BMD	94.5			5.5			
	Disk	94.5	15–27	0	5.5	14–NZ	0	100
	Tablet-MGM	96.4	19–38	0	3.6	20–NZ	2	98.2
	Tablet-RPMI	96.4	17–32	0	3.6	23–NZ	2	98.2
	Tablet-SHA	99.1	15–27	0	0.9	19–NZ	5	95.5

[a] BMD, MICs in μg/ml determined at 24 h (caspofungin for *Candida* spp.) to 48 h (other agent for *Candida* spp.) and 72 h (both agents for *C. neoformans*) by the CLSI M27-A2 microdilution method; Disk, inhibition zone diameters in mm determined at 24 h (*Candida* spp.) and 72 h (*C. neoformans*) by a modified M44-A method; Tablet-MGM, Tablet-RPMI, and Tablet-SHA, inhibition zone diameters in mm determined at 24 h (*Candida* spp.) and 72 h (*C. neoformans*) by the Neo-Sensitabs tablet method with MGM, RPMI 1640, and Shadomy agars, respectively. NZ, no inhibition zones around the tablet or disk.

[b] Percentages of MICs and inhibition zone diameters in mm that were within the assigned tentative MICs and zone diameter breakpoints in this study: susceptible, MIC of ≤1 μg/ml (≥14 mm for caspofungin and ≥15 mm for amphotericin B); resistant, MIC of ≥2 μg/ml (≤13 mm for both agents).

[c] Percentages of inhibition zone diameters in mm compared with the reference MICs that were in agreement regarding the tentative breakpoint classifications.

[d] COD and CBD, two disks (Oxoid and Becton Dickson, respectively).

were identified by both methods, and the level of agreement by "breakpoint category" with the microdilution method was high.

Due to methodology problems (narrow gap between amphotericin B MICs for susceptible and resistant strains), neither disk nor MIC breakpoints are available for this agent. An amphotericin B MIC of ≥2 μg/ml has suggested clinical resistance; it approximates achievable serum concentrations after high amphotericin B doses (≥1 mg/kg of body weight per day) and exceeds achievable cerebrospinal fluid concentrations. This study included four well-documented amphotericin B-resistant isolates (in vitro and in vivo data) (10). As for the caspofungin analysis, we categorized these four isolates and one isolate each of *C. albicans* and *C. krusei* as being resistant (Etest and broth dilution MICs of 2 to >8 μg/ml and corresponding zone diameters of ≤14 mm). Other isolates were categorized as being susceptible (MICs of ≤1 μg/ml and corresponding zone diameters of ≥15 mm; most MICs were also below 1 μg/ml by Etest) (Table 5). The disk method was able to identify the six resistant *Candida* isolates (zone diameters, 0 to 14 mm), but the Neo-Sensitabs assay failed to recognize two (MGM and RPMI agars) to five (SHA agar) of these isolates ("very major errors"). Therefore, disk testing appears to be a potential alternative to broth dilution methodology for yeast isolates with amphotericin B, but the tablet assay does not appear to be as promising or may require more development work. However, routine testing with this agent is not recommended until the controversial issue of establishing breakpoints for amphotericin B is resolved. Recent attempts to correlate in vitro results (Etest and CLSI methods) to clinical outcome for *Candida* bloodstream isolates failed to find a significant correlation (15).

In summary, based on reproducibility, the agreement by breakpoint category, and correlation coefficient data, the optimal testing conditions for the Neo-Sensitabs tablet assay for testing fluconazole and voriconazole appear to be those described by CLSI method M44-A for disk testing (MGM agar after 24 h of incubation for *Candida* spp. and 72 h for *C. neoformans*). This observation also applies to itraconazole and caspofungin disk and Neo-Sensitabs tablet testing. Further work is required to improve the testing of amphotericin B by the Neo-Sensitabs assay against yeasts since the correlation was low, and this method was unable to identify some well-documented amphotericin B-resistant isolates. Overall, the Neo-Sensitabs assay may be an alternative method for use in the clinical laboratory to determine the susceptibility of yeasts to four of the five agents evaluated. Collaborative studies to establish reference QC zone diameters and interpretive zone diameters for caspofungin and itraconazole as well as interpretive MIC breakpoints for caspofungin are required to better evaluate the suitability of this method.

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REFERENCES

1. **Barchiesi, F., E. Spreghini, S. Tomassetti, A. D. Vittoria, D. Arzeni, E. Manso, and G. Scalise.** 2006. Effects of caspofungin against *Candida guilliermondii* and *Candida parapsilosis*. Antimicrob. Agents Chemother. **50:**2719–2727.
2. **Cantón, E., J. Pemán, A. Carrillo-Muñoz, A. Orero, P. Ubeda, A. Viudes, and M. Gobernado.** 1999. Fluconazole susceptibilities of bloodstream *Candida* sp. isolates as determined by National Committee for Clinical Laboratory Standards method M27-A and two other methods. J. Clin. Microbiol. **37:**2197–2200.
3. **Casals, J. B.** 1979. Tablet sensitivity testing of pathogenic fungi. J. Clin. Pathol. **32:**719–722.
4. **Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution susceptibility testing of yeasts: approved standard, 2nd ed. Document M27-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards.** 2004. Method for antifungal disk diffusion susceptibility testing of yeasts: approved guideline. Document M44-A. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Clinical and Laboratory Standards Institute.** 2006. Quality control minimal inhibitory concentration (MIC) limits for broth microdilution and MIC interpretive breakpoints. Supplement M27-S2. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Clinical and Laboratory Standards Institute.** 2006. Zone diameter interpretive standards and corresponding minimal inhibitory concentration (MIC) interpretive breakpoints. Supplement M44-S1. Clinical and Laboratory Standards Institute, Wayne, PA.
8. **Espinel-Ingroff, A.** 1988. Comparison of in vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J. Clin. Microbiol. **36:**2950–2956.
9. **Espinel-Ingroff, A.** 2006. Comparison of three commercial assays and a modified disk diffusion assay with two broth microdilution reference assays for testing zygomycetes, *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans* with posaconazole and amphotericin B. J. Clin. Microbiol. **44:**3616–3622.
10. **Lozano-Chiu, M., P. W. Nelson, M. Lancaster, M. A. Pfaller, and J. H. Rex.** 1997. Lot-to-lot variability of antibiotic medium 3 used for testing susceptibility of *Candida* isolates to amphotericin B. J. Clin. Microbiol. **35:**270–272.
11. **Lozano-Chiu, M., P. W. Nelson, V. L. Paetznick, and J. H. Rex.** 1999. Disk diffusion method for determining susceptibilities of *Candida* spp. to MK-0991. J. Clin. Microbiol. **37:**1625–1627.
12. **Matar, M. J., L. Ostrosky-Zeichner, V. L. Paetznick, J. R. Rodriguez, E. Chen, and J. H. Rex.** 2003. Correlation between E-test, disk diffusion, and microdilution methods for antifungal susceptibility testing of fluconazole and voriconazole. Antimicrob. Agents Chemother. **47:**1647–1651.
13. **Odds, F. C., M. Motyl, R. Andrade, J. Bille, E. Canton, M. Cuenca-Estrella, A. Davidson, C. Durussel, D. Ellis, E. Foraker, A. W. Fothergill, M. A. Ghannoum, R. A. Giacobbe, M. Gobernado, R. Handke, M. Laverdiere, W. Lee-Yang, W. G. Merz, L. Ostrosky-Zeichner, J. Peman, S. Perea, J. R. Perfect, M. A. Pfaller, L. Proia, J. H. Rex, M. G. Rinaldi, J.-L. Rodriguez-Tudela, W. A. Schell, C. Shields, D. A. Sutton, P. E. Verweij, and D. W. Warnock.** 2004. Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. J. Clin. Microbiol. **42:**3475–3482.
14. **Ostrosky-Zeichner, L., J. H. Rex, P. G. Pappas, R. J. Hamil, R. A. Larsen, H. W. Horowitz, W. G. Powderly, N. Hyslop, C. A. Kauffman, J. Cleary, J. E. Mangino, and J. Lee.** 2003. Antifungal susceptibility survey of 2,000 bloodstream *Candida* isolates in the United States. Antimicrob. Agents Chemother. **47:**3149–3154.
15. **Park, B. J., B. A. Arthington-Skaggs, R. A. Hajjeh, N. Iqbal, M. A. Ciblak, W. Lee-Yang, M. D. Hairston, M. Phelan, B. D. Plikaytis, A. N. Sofair, L. H. Harrison, S. K. Fridkin, and D. W. Warnock.** 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. Antimicrob. Agents Chemother. **50:**1287–1292.
16. **Pfaller, M. A., L. Boyken, R. J. Hollis, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2006. In vitro susceptibilities of *Candida* spp. to caspofungin: four years of global surveillance. J. Clin. Microbiol. **44:**760–763.
17. **Pfaller, M. A., D. J. Diekema, S. A. Messer, L. Boyken, and R. J. Hollis.** 2003. Activities of fluconazole and voriconazole against 1,586 recent clinical isolates of *Candida* species determined by broth microdilution, disk diffusion, and Etest methods: report from the ARTEMIS global antifungal susceptibility program. J. Clin. Microbiol. **41:**1440–1446.
18. **Pfaller, M. A., D. J. Diekema, M. G. Rinaldi, R. Barnes, B. Hu, A. V. Veselov, N. Tiraboschi, E. Nagy, D. L. Gibbs, and the Global Antifungal Surveillance Group.** 2005. Results from the ARTEMIS disk global antifungal surveillance study: a 6.5-year analysis of susceptibilities of *Candida* and other yeast

species to fluconazole and voriconazole by standardized disk diffusion testing. J. Clin. Microbiol. **43:**5848–5859.
19. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2004. Evaluation of NCCLS M44-P disk diffusion method for determining susceptibilities of 276 clinical isolates of *Cryptococcus neoformans* to fluconazole. J. Clin. Microbiol. **42:**380–383.
20. **Rubio, M. C., J. Gil, I. Ramirez de Ocariz, R. Benito, and A. Rezusta.** 2003. Comparison of results obtained by testing with three different agar media and by the NCCLS M27-A method for in vitro testing of fluconazole against *Candida* spp. J. Clin. Microbiol. **41:**2665–2668.
21. **Sandven, P.** 1999. Detection of fluconazole-resistant *Candida* strains by a disc diffusion screening test. J. Clin. Microbiol. **37:**3856–3859.
22. **Vandenbossche, I., M. Vaneechoutte, M. Vandevenne, T. De Baere, and G. Verschraegen.** 2002. Susceptibility testing of fluconazole by the NCCLS broth microdilution method, E-test, and disk diffusion for application in the routine laboratory. J. Clin. Microbiol. **40:**918–921.